



## Feminization tendency of Hawksbill Turtles (*Eretmochelys imbricata*) in the western Yucatán Peninsula, Mexico

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**Abstract.**—The viability of sea turtle populations during their early stages depends mostly on hatching success and a balanced sex ratio of the hatchlings. In sea turtles, sex is determined by the temperature at which the eggs are incubated. Consequently, climate change can play a critical role in population fitness, because increased temperatures can skew the sex ratio towards females. The Hawksbill Turtle is a Critically Endangered species and Mexico holds one of the largest populations of nesting females worldwide. Given the importance of Mexican Hawksbill populations, it is necessary to evaluate their nest temperatures, particularly during the thermosensitive period, and identify the sex ratio of the hatchlings. Hawksbill Turtle nests were characterized in three nesting beaches from the western Yucatán Peninsula, Mexico, to determine the temperatures of the nests *in situ* and the sex ratio of the hatchlings. The results showed that the incubation temperature was warmer than the pivotal temperature by  $1.255 \pm 0.18$  (SE) °C, and in 82.05% of the nests monitored with only female hatchlings. This was also confirmed via histology of the gonads of dead hatchlings. The amount of shade above the nests and rainfall were the most influential factors for decreasing nest temperatures. This study shows a trend toward the feminization of hatchlings due to the low percentage of shade above nests which is able to increase the sex ratio bias due to coastal dune deforestation, together with the increase in environmental temperatures due to climate change. The skewed ratio toward females could have negative consequences for the maintenance of sea turtle populations on the Yucatán Peninsula, so is necessary to develop strategies of conservation in order to reduce this trend.

**Keywords.** Anthropogenic impact, climate change, coastal-dune vegetation, female hatchlings, gonad histology, nest temperature, pivotal temperature, rain, shade, temperature-dependent sex determination

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### Introduction

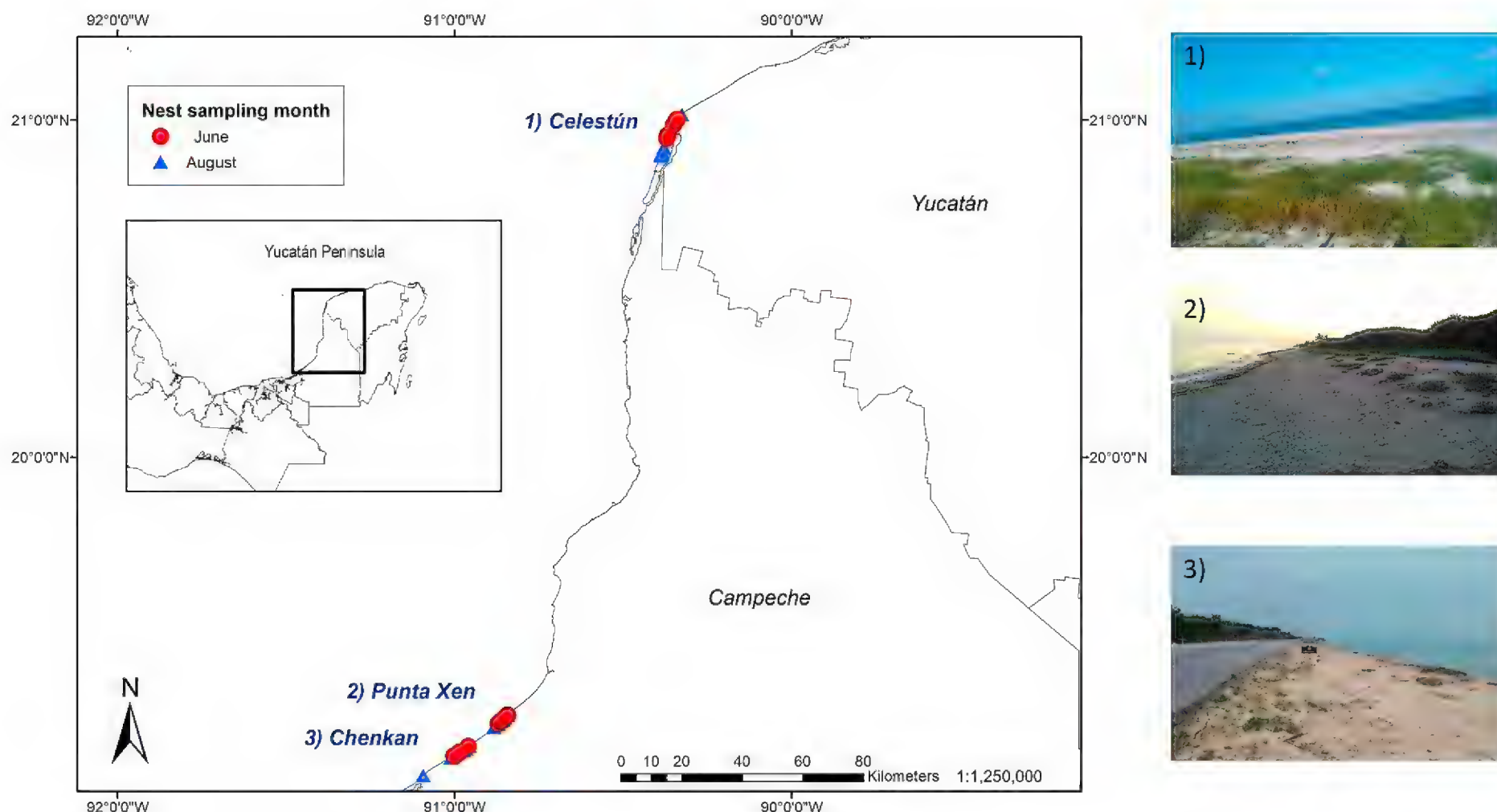
Temperature-dependent sex determination occurs in a variety of reptiles, including all sea turtles (Bull and Vogt 1979; Wibbels 2003). In these species, the temperature that embryos experience during their development determines the sex of hatchlings. Specifically, sex determination occurs in the thermosensitive period (TSP), which is a particular time during embryonic development (the middle third of incubation) when the temperature affects gonad differentiation (Ciofi and Swingland 1997; Mrosovsky and Pieau 1991). The TSP provides a balance between both sexes at a certain temperature, called the

pivotal temperature (PT), and increasing or decreasing the temperature relative to the PT shifts the sexual proportion towards one sex (Mrosovsky and Yntema 1980). In sea turtles, the sex ratio is skewed toward males at temperatures below the PT and toward females at temperatures above the PT (Salame-Méndez 1998). Since the PT for all sea turtles is between 28 °C and 30 °C (Hawkes et al. 2009), the increase in temperature due to global warming of the planet could skew the sex ratio.

A bias towards feminization has been reported in all species of sea turtles in multiple studies around the world since 1997 (Hawkes et al. 2009), being less represented in Hawksbill Turtles (*Eretmochelys imbricata*; Hawkes

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**Fig. 1.** Geographical locations of the monitored nests of *Eretmochelys imbricata*, as well as the beaches studied and their images. 1) Celestún, 2) Punta Xen, and 3) Chenkan.

et al. 2009; Hays et al. 2017); even though Hawksbills are considered Critically Endangered (see <https://www.iucnredlist.org/search?taxonomies=100888&searchType=species>). Thus far, no studies have addressed incubation temperatures on Mexico's nesting beaches, even though the Yucatán Peninsula hosts one of the largest populations of Hawksbill females worldwide and the largest nesting population of Hawksbill Turtles in the Atlantic (Campbell 2014; Garduño-Andrade and Guzmán 1999; Meylan and Donnelly 1999; Mortimer and Donnelly 2008).

Sea turtles select microenvironments for nesting, thereby manipulating the thermal conditions to which their eggs will be exposed during incubation (Difffenbaugh and Field 2013). Thus, the nest temperature will be modified according to its location with respect to several factors, including vegetation (Kamel 2013; Kamel and Mrosovsky 2006), distance to the coastline (Rees and Margaritoulis 2004), sand differences in albedo and grain size (Hays et al. 2001; Schaetzel and Anderson 2005), and others. In addition, the thermal properties of the beaches can provide different incubation temperatures (Hawkes et al. 2009), and the temperature varies across the nesting season (Mrosovsky et al. 1984).

Therefore, this study was carried out on three beaches in the western Yucatán Peninsula with different degrees of anthropogenic impact (disturbance due to human infrastructures like roads, building for tourists, vegetation removal, etc.) as well as variations in the littoral characteristics (width of the beaches, distribution and type of natural vegetation). These differences are expected to promote diverse thermal gradients which modify the nest temperatures, and, consequently, sex ratios and hatching success. It is known that the least

preserved beaches (especially in the dune strip) are those that maintain the highest average temperatures and decrease the hatching success (Pike 2008, 2009), particularly for Hawksbill Turtles whose nesting is closely related to vegetation (Horrocks and Scott 1991; Kamel 2013). Therefore, the objectives of this study were to determine the temperatures of Hawksbill Turtle nests *in situ* during the TSP and estimate their sex ratios based on a histological calibration, as well as determine if there are differences in the sex ratio over the course of the nesting season and among beaches.

## Materials and Methods

Fieldwork was carried out on three index nesting beaches (historical localities with higher nesting females) of the Hawksbill Turtle in the western Yucatán Peninsula, Mexico. The beaches were: Chenkan ( $19^{\circ}07'50''\text{N}$ ;  $90^{\circ}31'13.15''\text{W}$ ) and Punta Xen ( $19^{\circ}12'42.57''\text{N}$ ;  $90^{\circ}52'07.01''\text{W}$ ) in the state of Campeche, and Celestún ( $20^{\circ}59'33.72''\text{N}$ ;  $90^{\circ}24'54''\text{W}$ ) in the state of Yucatán (Fig. 1). These beaches were selected as they present differences in their littoral characteristics (width of the beach and type of vegetation) and in their conservation status (degree of disturbance due to human infrastructures). Celestún is the most well-preserved beach (with wider beaches, low disturbance in vegetation, limited erosion and few human infrastructures on most of the beach), while Chenkan is the least preserved (with high erosion and deforestation rates, and a road along the beach). The study was conducted in 2017, during the middle (June–July) and end (August–September) of the nesting season. June is the month when the nesting increases

(Guzmán et al. 2008), so it would be more representative to sample then, while in August the temperature begins to decrease due to the increase in rainfall (García 2004). These differences could allow the evaluation of distinct responses to the temperature during nesting, which would modify the sex of the hatchlings and would therefore provide greater variability in the samples.

The permit to handle the Hawksbill Turtles was provided by SEMARNAT (SGPA/DGVS/03995/17). The fieldwork consisted of making routes from 2100 to 0500 h, searching for nesting Hawksbill Turtles randomly but trying to include different thermal zones (i.e., under high vegetation (HV), under low vegetation (LV), and without vegetation (WV), at different distances along each beach). The study was conducted using only nests *in situ* (i.e., places that females naturally selected to lay their eggs), so once the turtles began to oviposit, the nest location data were taken with a Global Positioning System (GPS), as the nests were left exactly where the turtles nested.

The characterization of each nest involved the following procedure. The temperatures (°C), both inside and outside the incubation chamber, were taken with a digital thermometer (Fluke, 51-II®). The depth of the nest from the first to the last egg deposited was measured with a flexometer. Temperature data loggers (HOBO® pendant) were placed in the middle of the nests and temperature data were recorded every half-hour throughout the incubation period. The distances from the nest to the nearest vegetation and to the coastline were measured with a flexometer. The type of vegetation closest to the nest was recorded, and the percentage of shade above the nest was established when checking the nest close to noon.

The hatching success was determined according to Miller (1999), by checking the nests once the hatchlings had left, after  $55 \pm 5$  d incubation (Guzmán 2008). At the hatchling stage, sea turtles cannot be sexed by external morphology (Merchant 1999; Wibbels 2003). Therefore, the sex ratios of the Hawksbill hatchlings were estimated using the temperatures recorded by the data logger during the TSP, which were analyzed by the TSD software version 4.0.3 designed by Girondot (1999). In addition, the temperature used to determine the sex in the software was calibrated based on the histology of the dead hatchlings, as this option avoided destructive sampling of this Critically Endangered species. Any dead hatchlings found in the monitored nests were collected to dissect the urogenital complex (mesonephros and gonads), which was fixed in 4% paraformaldehyde and transported to the Laboratorio de Embriología de la Facultad de Medicina, Universidad Nacional Autónoma de México. In the laboratory, the samples were dehydrated by gradual ethanol solutions and maintained in 75% ethanol. Later, they were embedded in histological paraffin and the gonads were sectioned (between 8 and 12  $\mu\text{m}$  thick) using a rotary microtome. Subsequently,

the samples were stained with periodic acid-Schiff's reaction and mounted on slides for observation under an optical microscope. Under the microscope, the ovaries or testes were identified using the criteria of Yntema and Mrosovsky (1980) and Wibbels et al. (1999).

Sex ratio results obtained from gonad histology were used as input for the TSD software to obtain a field estimate of the PT for this species. Subsequently, this PT was used as a formula parameter for estimating the hatchling's sex ratios, based on the mean incubation temperature during TSP recorded in each nest. Since the TSD software was developed for use with data derived from constant temperature incubations, the average temperature (and standard deviation) of each nest was measured during each third of the incubation period, in order to gauge the temperature variability within the nests.

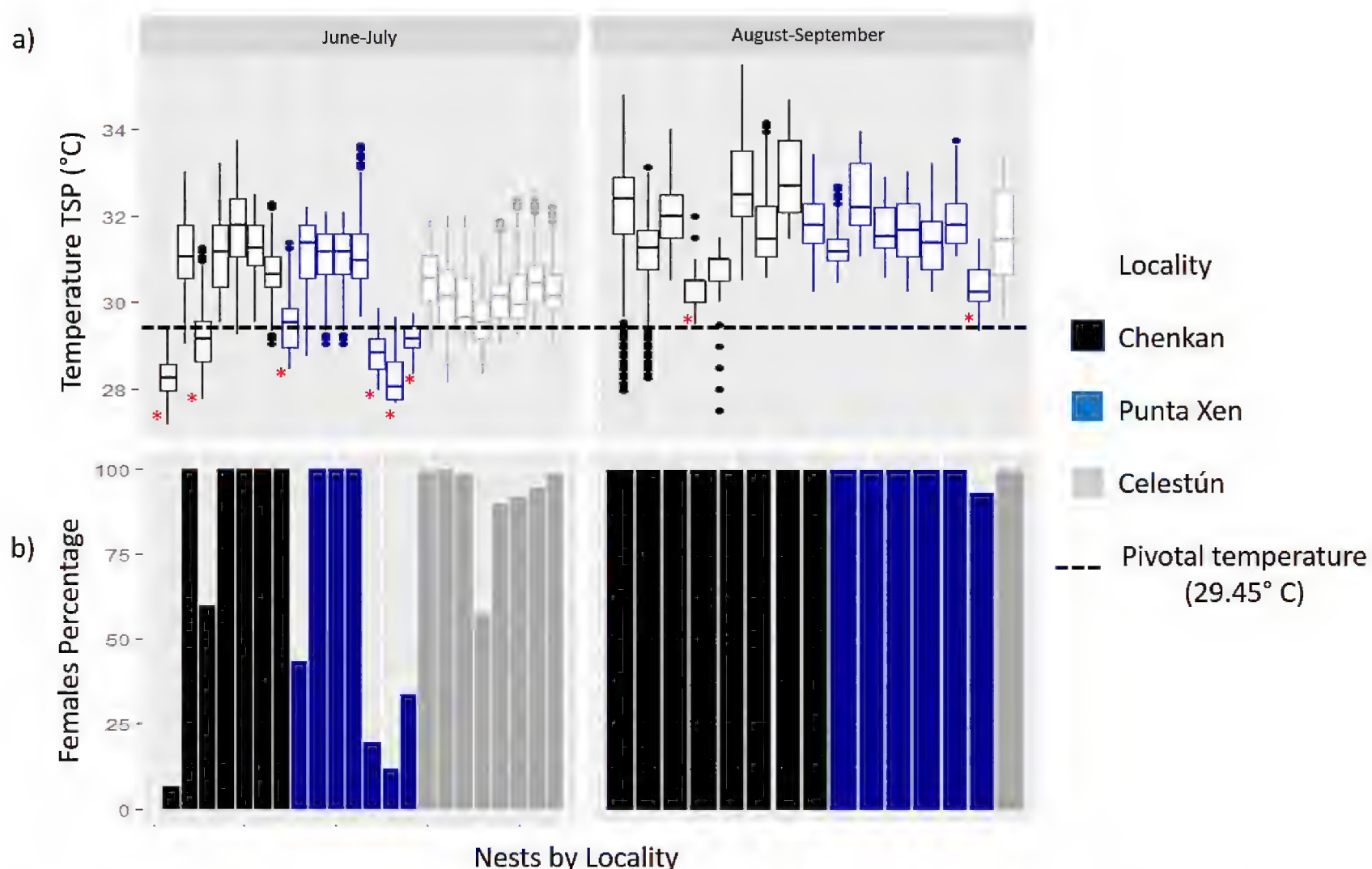
Since the shade provided by the HV decreases the temperature of the nests (Kamel 2013; Kamel and Mrosovsky 2006), the percentages of nests that would have temperatures close to the PT (shaded nests) and nests that would have feminizing temperatures (sun-exposed nests and those under LV) were estimated according to the thermal zones (HV, LV, and WV) along each beach. The thermal zones of the beaches were categorized based on the width (distance from the coastline to the HV or limit where turtles could not nest, such as a human infrastructure) and the dominant vegetation every 2 km on Chenkan (18 km) and Punta Xen (18 km) beaches, and every 5 km on Celestún (22 km). These measurements of the beaches, together with the geographical locations recorded for the nests, were used to correlate them with satellite images and the maximum distance of the nests from the coastline (according to our results), through Google images, in each beach per km (in linear m).

To document the degree of anthropogenic impact on each beach, Google satellite images were used to measure (in linear m) the disturbance along each beach, and thereby estimate the percentage of human infrastructures that would affect the nesting of the turtles per km. To establish the number of nests per km throughout the entire nesting period of the Chenkan and Punta Xen beaches, databases were obtained from the Comisión Nacional de Áreas Naturales Protegidas (CONANP) and Grupo Ecologista Quelonios A.C., respectively. The precipitation levels on the three beaches during the entire nesting period were obtained from the Comisión Nacional del Agua (CONAGUA), in order to relate the dates of both incubation periods with the amount of rain.

### Statistical Analysis

The normality and homogeneity of the data were tested by Kolmogorov-Smirnov and Levene's tests. Wilcoxon tests (as the distribution was not normal) were used to compare the nest temperatures, hatching success, and the variables that characterize the nests (incubation





**Fig. 2.** (a) Incubation temperatures of *Eretmochelys imbricata*, during the TSP, on the beaches of Chenkan, Punta Xen, and Celestún, during two incubation periods (June–July and August–September). The dotted line indicates the pivotal temperature. (b) Percentages of females in the monitored nests on the nesting beaches of the western Yucatán Peninsula (Mexico). The red asterisks show where the differences were significant ( $p < 0.05$ ) among nests, by beach.

chamber temperature, depth, distance to the vegetation and to the coastline, as well as percentage of shade above the nests) between the two incubation periods (June–July and August–September) within each beach. An ANOVA was performed to compare the different variables that characterize the nests among the three monitored beaches. To determine the TSP of each nest, the days from the beginning to the end of incubation were calculated, and the middle third of the incubation period was established. Subsequently, the temperature during the TSP was compared among the beaches by a Generalized Linear Model (GLM), with the set of nest temperatures at each beach as the response variable. To compare the temperatures during the TSP among the nests on each beach, a GLM was used, with the temperatures recorded for each nest as the response variable. In addition, a time series was used, where nest temperatures were correlated with the time at which they were taken, and were compared among all the nests for each locality. Measuring these correlations established the time required for shaded nests to absorb heat, in comparison to sun-exposed nests. Statistical significance was set at  $p < 0.05$ ; and SPSS, Stata SE11, and R version 3.5.1 (R Core Team 2018) were used for statistical analyses.

## Results

During the 2017 nesting season, 20 nests were characterized on Chenkan beach (10 in June and 10 in August), 20 on Punta Xen beach (10 in June and 10 in

August), and 12 on Celestún beach (eight in June and four in August). The nests characterized along the three monitored beaches showed no significant differences in the temperature of the incubation chamber, distance to the coastline, or percentage of shade ( $F_2 = 1.614$ ,  $p = 0.214$ ). However, the distance to the nearest vegetation differed significantly among beaches, with nests in Celestún being the farthest from vegetation ( $F_2 = -3.514$ ,  $p = 0.042$ ). Likewise, nest depth differed significantly among the beaches, with the nests on Chenkan beach being the most shallow ( $F_2 = 3.85$ ,  $p = 0.036$ ; Table 1).

The average temperatures of the monitored nests did not vary significantly among the three beaches during June–July ( $F_2 = 0.202$ ,  $p = 0.819$ ), nor in August–September ( $F_2 = 0.462$ ,  $p = 0.640$ , Table 2). The average incubation temperature on Chenkan beach was higher in August–September ( $31.89 \pm 1.36$  °C) than in June–July ( $30.78 \pm 1.08$  °C,  $F_{11,73} = 2.246$ ,  $p = 0.045$ ). In Punta Xen, only the average temperature during the TSP was higher in August–September ( $31.386 \pm 1.164$  °C) than in June–July ( $29.869 \pm 0.728$  °C,  $F_{10,18} = 2.857$ ,  $p = 0.017$ ; Table 2). The incubation temperatures across all beaches and the entire nesting season were within the thermal interval that allows embryonic development (28.5 to 32.9 °C, Table 2, Fig. 2). The nest temperatures recorded during the TSP of June–July at Chenkan beach showed significant differences among them, according to their locations ( $F_7 = 34.830$ ,  $p < 0.001$ ), and the same was found at Punta Xen beach ( $F_7 = 49.911$ ,  $p < 0.001$ ). For the nests that were found under the shade, more than



**Table 1.** Nest characteristics of Hawksbill Turtles in the western Yucatán Peninsula, Mexico.

Locality	Nesting (month)	Internal temperature (°C)	External temperature (°C)	Total depth (cm)	Depth of the last egg (cm)	Distance to vegetation (m)	Distance to coastline (m)	Percentage of shade (%)
Chenkan	June	29.73 ± 1.49	26.8 ± 1.29	32.1 ± 6.85	14.73 ± 7.16	1.15 ± 1.86	17.79 ± 5.09	36.67 ± 46.90
	August	29.38 ± 1.94	26.62 ± 2.54	44.75 ± 7.18	25.41 ± 8.51	0.49 ± 1.48	15.06 ± 2.43	9.57 ± 19.12
Punta Xen	June	29.19 ± 0.59	27.40 ± 0.86	34.58 ± 6.72	21.13 ± 3.98	0.86 ± 1.50	13.04 ± 10.07	46.11 ± 15.95
	August	28.3 ± 1.70	27.57 ± 1.15	46.45 ± 7.56	28.65 ± 7.81	1.78 ± 3.52	19.78 ± 6.53	24.44 ± 34.68
Celestún	June	28.62 ± 0.55	26.84 ± 1.12	37.57 ± 6.03	20.93 ± 4.44	4.07 ± 4.68	19.74 ± 6.09	26.67 ± 15.28
	August	28.78 ± 0.46	26.98 ± 1.27	49.67 ± 4.62	35.33 ± 7.51	2.45 ± 0.97	20.48 ± 5.53	22.5 ± 10.61

70% of their total coverage throughout the incubation period had significantly lower temperatures compared to sun-exposed nests ( $p < 0.001$ , Fig. 2a). On Celestún beach, the nest temperatures did not show significant differences between these two conditions of shaded (with less than 50% of total coverage) and sun-exposed. The nest temperatures in the TSP of August–September at Chenkan beach showed significant differences only in one nest, with lower temperatures compared to the other nests ( $F_7 = 11.320$ ,  $p < 0.001$ ), and the same pattern occurred on Punta Xen beach ( $F_7 = 13.493$ ,  $p < 0.001$ ). Both of these nests on each beach were sun-exposed, therefore they remained above the PT (Fig. 2a). The nests that were found under total shade coverage throughout the incubation period, exhibited a temperature damping effect, taking 3.5–4 h longer to heat up to the same temperature as the sun-exposed nests ( $p < 0.001$ ). Although the shaded nests had lower temperatures than those that were sun-exposed, most of the nests were exposed; and therefore, in general, the nest temperature during the TSP was hotter than the PT.

The hatching success for June–July did not differ significantly between the three beaches: Chenkan (61.9 ± 26.71%), Punta Xen (57.97 ± 41.04%), and Celestún (71.38 ± 24.53%,  $F_2 = 0.54$ ,  $p = 0.589$ ). A similar trend was observed in the period of August–September on Chenkan (50.89 ± 24.36%), Punta Xen (26.02 ± 36.3%), and Celestún (61.95 ± 41.94%,  $F_2 = 2.30$ ,  $p = 0.125$ ), although numerically lower hatching success was observed in the nests located in Punta Xen (Fig. 3).

During the cleaning of the nests in both nesting periods, 170 dead hatchlings were collected from 17 nests (five from Chenkan, five from Punta Xen, and seven from Celestún). In a nest from Chenkan beach (June–July), a sample of 31 dead hatchlings was obtained, which were predated by ghost crabs. In addition, on Punta Xen beach

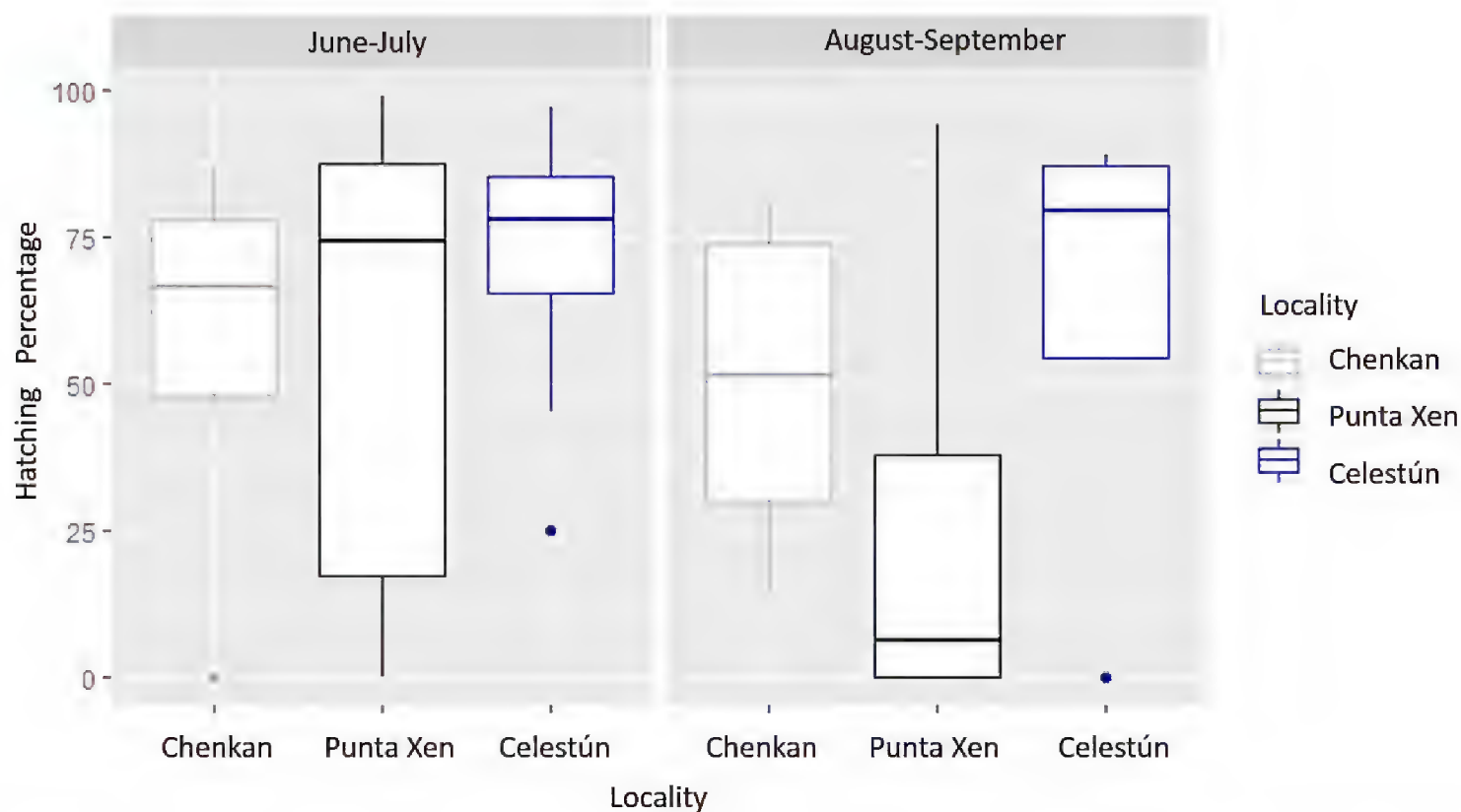
(August–September), a sample of 65 dead hatchlings was obtained, which were predated by ants (*Solenopsis germinata* and *Labidus coecus*). From these two nests, the largest and freshest samples were obtained because the hatchlings were found less than 12 h after death. In five nests, the hatchlings required more than 36 h to emerge from the nests, therefore the tissues of dead hatchlings were degraded and the sexes of 25 samples were unidentifiable. In total, 145 gonads from 12 nests were identified. Histological examination showed that the ovaries of hatchlings exhibited a thickened cortex and unorganized medulla, whereas testes presented a very thin cortex and had an organized medullary region with developing seminiferous cords (Fig. 4). From the total sample, 126 females and 19 males were determined. Only hatchling females were found in the sun-exposed nests, with temperatures of 30.21 °C and above. However, both sexes were found in the shaded nests, with temperatures in the range of 28.55–29.59 °C (Table 3). The temperature variation in the nests during the TSP, from which the histological samples were obtained, was  $0.77 \pm 0.15$  °C. These results were used as the input and for calibration of the TSD software, which determined the pivotal temperature as  $29.45 (\pm 0.147 \text{ SE})$  °C, according to the maximum-likelihood analysis, with the Richard model ( $p_{(10)} < 0.001$ , AIC = 61.54, Fig. 5). In addition, the transitional range of the temperature was  $-0.53 \pm 0.048$ . According to the TSD software, only 27.02% of the hatchlings were males in the monitored nests. The highest percentage of females occurred during the late nesting season (August–September,  $83.33 \pm 4.01\%$ ) and the lowest percentage occurred during the middle (June–July,  $65.49 \pm 4.08\%$ ,  $t_{22,2} = -3.72$ ,  $p = 0.006$ ; Fig. 2b).

In the three studied beaches, the dominant vegetation was the coastal dune halophyte, which is mainly low and medium-sized, characterized by herbaceous plants

**Table 2.** Average nest temperatures during the total incubation period and the TSP of the Hawksbill Turtles for each monitored beach, between the months indicated. Significant differences ( $p < 0.05$ ) are indicated by asterisks.

Beach	Temperature during incubation (°C)		Temperature during TSP (°C)	
	June–July	August–September	June–July	August–September
Chenkan	30.78 ± 1.08*	31.9 ± 1.36*	30.49 ± 0.72*	31.71 ± 0.89*
Punta Xen	30.44 ± 1.44	31.36 ± 1.23	29.87 ± 0.62*	31.63 ± 0.68*
Celestún	30.85 ± 1.48	30.4 ± 0.95	30.29 ± 0.83	30.1 ± 0.63





**Fig. 3.** Hatching success of *Eretmochelys imbricata* on the beaches of Chenkan, Punta Xen, and Celestún, Yucatán Peninsula (Mexico).

and shrubs. However, the high vegetation (HV; i.e., the vegetation that provides more shade) changed among the three beaches. In Chenkan, the HV was dominated by spots of coconut plantations, present only in 22.2% of the total length of the beach, because natural vegetation (mangroves) was located on the other side of a road. In Punta Xen, the dominant HV was the mangroves, present in 62.5% of the total length of the beach; whereas in Celestún, the HV was dominated by tropical dry forest, which corresponded to 77.3% of the total length of the beach. Nevertheless, the percentage of shade provided by the HV to the nests was not equal to the percentage of HV along the three beaches studied, since it depended on the distance between the HV, the nests, and the coastline, as well as the degree of anthropogenic disturbance. Thus, according to the distance from the coastline to the HV and the maximum distance between the monitored nests and the coastline (30 m; Table 1) the percentage of the shaded nest was calculated. Chenkan beach had the lowest percentage of shaded nests, with only 2.89%, next was the Celestún beach with 21.54%, and the highest

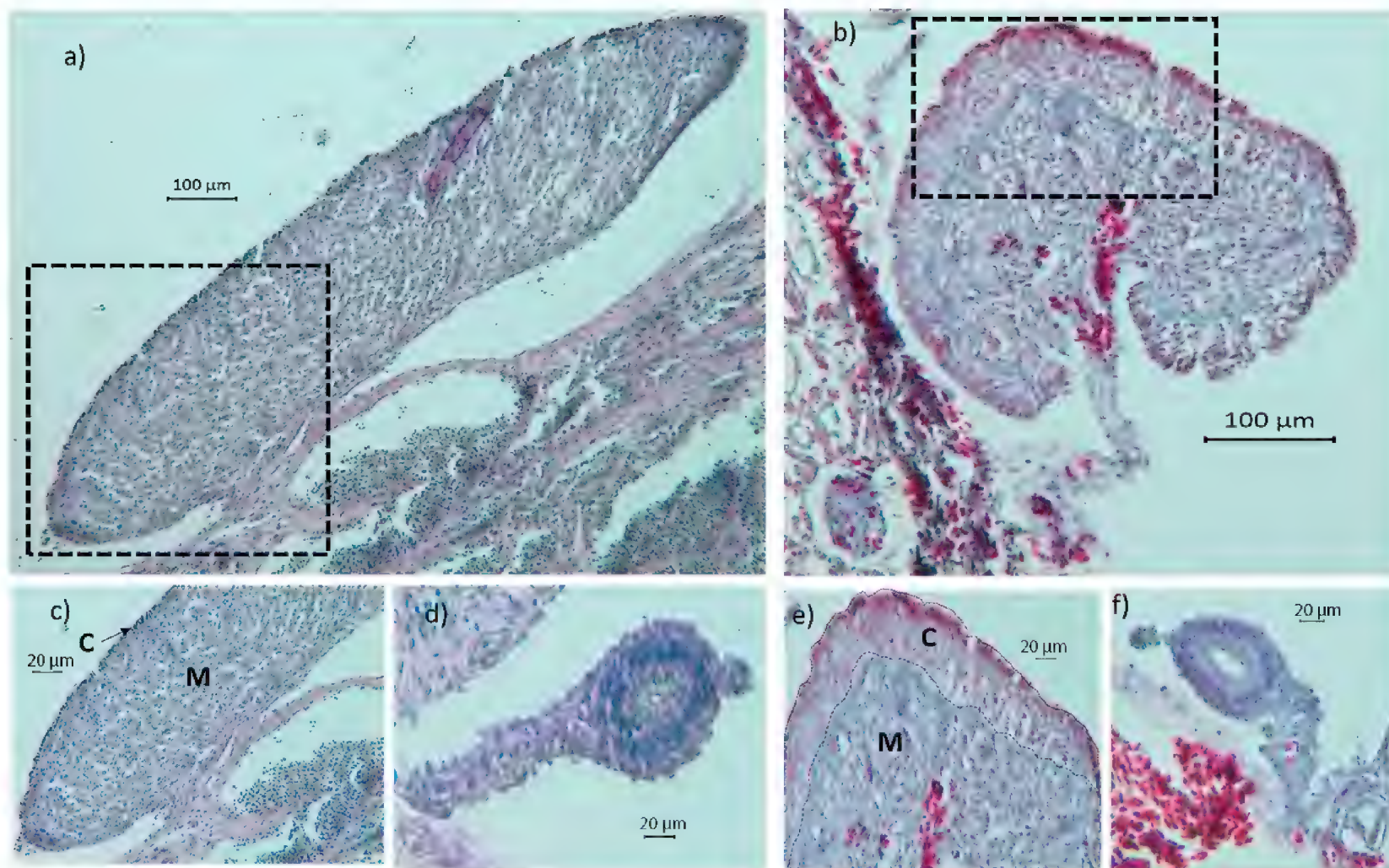
was the Punta Xen beach with 29.59%. The percentage of shade above the monitored nests on the three beaches also changed between the periods of June–July and August–September. In June–July, 50% of the nests on Celestún were recorded under shrubs, 50% of the nests in Punta Xen were under the shade of the mangroves, and 40% of the nests in Chenkan were under shade (coconuts and man-made structures to create shade on the beach, known as *palapas*). In contrast, in August–September, most of the nests were found in less shaded areas: in Celestún, 0% of the monitored nests were under shade, in Punta Xen, 20% nests were shaded, and in Chenkan, 10% of nests were shaded.

According to the analysis of anthropogenic impacts, Chenkan beach exhibited a 100% disturbance, mainly due to the road along the beach. On average, the distance was  $17.38 \pm 7.55$  m from the coastline to the road in the most eroded area, which caused the nesting of the turtles to be interrupted. In contrast, the wider area from the road to the coastline had an amplitude of 140.57 m, however, it had high deforestation and human infrastructures, such

**Table 3.** Numbers of dead hatchlings and percentages of females, according to the histological sex, temperature during the TSP at which nests were incubated *in situ*, and the percentage of shade above the nests, by locality and incubation period.

Period	Beach	Shade (%)	No. dead hatchlings	Temperature during TSP (°C)	Females (%)
June–July	Chenkan	100	3	$29.59 \pm 0.52$	67
		0	31	$30.66 \pm 0.44$	100
	Punta Xen	100	6	$28.55 \pm 0.66$	17
		90	8	$28.84 \pm 0.51$	25
		70	3	$29.35 \pm 0.59$	33
	Celestún	60	10	$29.6 \pm 0.64$	50
		20	2	$30.21 \pm 0.67$	100
40		3	$30.44 \pm 0.82$	100	
40		4	$30.93 \pm 0.94$	100	
August–September	Chenkan	40	2	$31.12 \pm 0.97$	100
	Punta Xen	0	65	$31.47 \pm 0.73$	100
		0	8	$32.41 \pm 0.89$	100





**Fig. 4.** Representative histology sections of gonads from dead Hawksbill hatchlings in the monitored nests. **(a)** Testis (100  $\mu\text{m}$ ), **(b)** Ovary (100  $\mu\text{m}$ ), **(c)** Testis (20  $\mu\text{m}$ ), **(d)** Müllerian ducts of male (20  $\mu\text{m}$ ), **(e)** Ovary (20  $\mu\text{m}$ ), **(f)** Müllerian ducts of female (20  $\mu\text{m}$ ). The dotted areas in **(a)** and **(b)** indicate the sites of the higher magnifications shown in **(c)** and **(e)**, where the dotted line indicates the cortex, **M** indicates the medulla region, and **C** indicates the cortex region.

as summer houses, restaurants, *palapas*, and fishing areas. In addition, there were breakwaters in the sea. Punta Xen beach had an average width of  $22.86 \pm 11.55$  m. The disturbance represented 14.95% of the length of the beach, mainly in places which were recreation areas, as well as areas with high deforestation rates and the presence of infrastructures, such as a hotel complex. Celestún was the most well-preserved beach, presenting only 6.03% disturbance, mainly due to the presence of two hotel complexes and some summer houses. The average width of the beach was  $42.59 \pm 6.69$  m.

According to the results, the percentage of shade above the nests was the main factor that decreased their temperatures, approaching a PT. Therefore, the percentage of shaded nests, the degree of conservation of the beaches, and the number of nests per km were used to estimate the percentage of nests with temperatures close to the pivotal for the 2017 nesting season. Chenkán beach had the greatest feminization among all the nests, showing only 0.5% of shaded nests. Punta Xen beach had the highest percentage of HV near the nesting areas, so it is estimated that 39.54% of the nests would have temperatures close to the pivotal (shaded nests). For Celestún beach, the HV was far from the nesting areas, so only 21.54% of the nests would have been under the shade.

With respect to the average rainfall, it differed between the two incubation periods on the beaches of Punta Xen and Chenkan. For June–July it was  $10.61 \pm 4.66$  mm, while for August–September it was  $7.22 \pm 2.68$  mm. In Celestún, precipitation was very low during both periods of incubation, without even reaching 1 mm

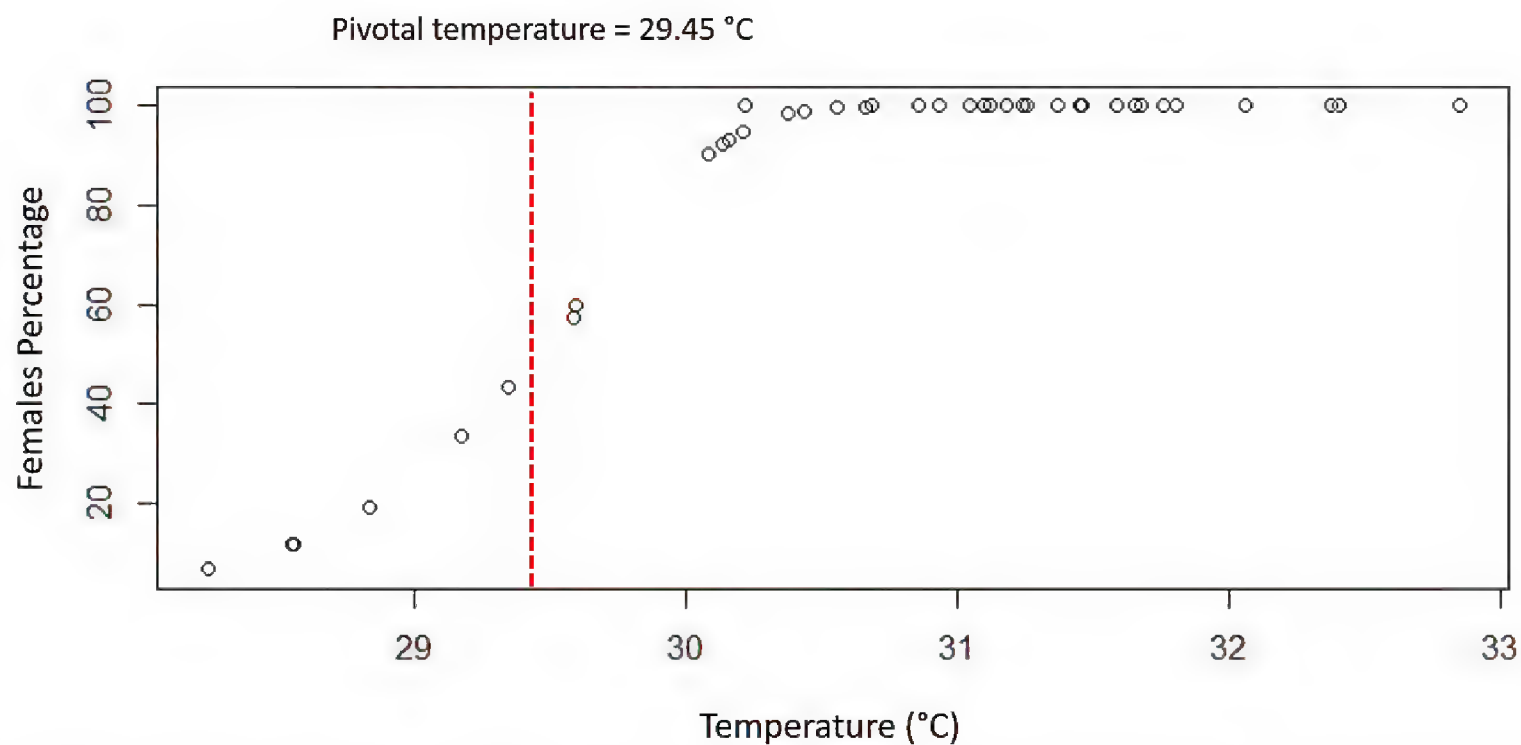
of precipitation, while the relative humidity remained between 74% and 76% for all incubation months, increasing to 95% in September.

## Discussion

In species with genetic sex determination systems, Fisher's theory predicts that the sex ratio in a population tends toward 1:1 (males:females). Given that when either sex is in the majority, the other sex has an advantage in finding mates, an equal sex ratio represents the evolutionarily stable strategy. However, in organisms with temperature-based sex determination this balance can become biased by warm or cold environments (Janzen and Phillips 2006).

The beaches have different environmental temperatures, which provide diverse thermal gradients to the nests (Diffenbaugh and Field 2013; Hawkes et al. 2009; Marcovaldi et al. 2014), so differences in incubation temperatures among nesting beaches would be expected. In the present study, despite the differences in littoral characteristics and conservation status among the three monitored beaches, and the distinct geographical position of Celestún relative to the other two beaches, no significant differences in incubation temperatures were found. This result may be due to the nesting characteristics of the Hawksbill Turtles, since most nests were found in the dune zone, which is associated with vegetation (Horrocks and Scott 1991), and nest temperatures are influenced by the type of vegetation present in each beach. Nevertheless, the low vegetation (LV) and the edge of the high vegetation (HV) do not provide lower





**Fig. 5.** Relationship between temperature and sex ratio. The red dotted line denotes the pivotal temperature according to the TSD software and calibrated with histology of gonads from dead Hawksbill hatchlings.

temperatures, and only the direct influence of the HV promotes lower temperatures and therefore more male hatchlings (Kamel 2013; Kamel and Mrosovsky 2006). On all three beaches, the vegetation in the nesting areas was low, which failed to decrease the temperatures. Also, the HV near the nests was scarce (less than 30% of the total nests by locality), which caused the majority of the nests to exhibit similar thermal conditions. However, the conditions of each beach differed in the factors that determine the nest temperatures. On Chenkan beach, the least HV occurred because of the high level of anthropogenic impact, primarily by the road that divided the nesting area from the native HV. As a consequence, nesting was prevented in the areas with lower temperatures (below the mangrove), which caused the majority of the nests to be exposed to feminizing temperatures. In contrast, although Celestún was the most well-preserved beach, the HV was generally found inland from the coastal dune, so the distance between the zone of HV and the coastline was wide. On all three beaches, the distance from the nests to the coastline was an average of  $\sim 17.83$  m, and most turtles nested in the dunes. As a consequence, few turtles reached the zone of HV, which was located at an average of  $\sim 45.59$  m from the coastline. Therefore, the majority of turtles nested in areas near LV, so most of the nests would have been in feminizing temperatures. Punta Xen beach had patches with HV (mangroves), so it was the beach with the highest percentage of shaded nests. Thus, the incubation temperatures among the nests were diverse, generating the highest percentage of nests close to the PT among the three monitored beaches. However, in this camp the nests are routinely translocated to hatcheries, due to high predation, so it is not known how this action would affect the sex ratio of the hatchlings. Therefore, despite the differences in the beaches, the trend toward feminization of Hawksbill Turtle hatchlings remained. Unfortunately, in the western Yucatán peninsula, the environmental temperatures are warmer now than 40 years ago (Sinervo

et al. 2010), and they also are in the studied beaches ( $\sim 2.33$  °C; CONAGUA). Even though vegetation can play an important role in reducing the nest temperatures, as environmental temperatures continue to rise, the trend toward feminization of hatchlings will increase.

Sea turtles lay several nests over a nesting season, so the environmental temperatures trigger differences in the sex ratio, with a higher percentage of female hatchlings produced in the middle of the nesting period (Mrosovsky et al. 1984). The seasonality in our study area also modified the nest temperatures and, therefore, the sex ratio of the hatchlings. However, contrary to the study of Mrosovsky et al. (1984), we found higher temperatures ( $\sim 31.89$  °C) at the end of the nesting season (August–September) than during the middle (June–July,  $\sim 30.78$  °C). These lower temperatures were due to the early rains (June–July) that reduced the nesting temperature and increased the proportion of male hatchlings (Wyneken and Lolavar 2015). In addition, the rain causes an increase in foliage, so most of the nests in June–July were shaded, which influenced the temperature difference between the two incubation periods.

Hatching success did not vary significantly among the beaches and months studied. In contrast, Pike (2008, 2009) previously found that beaches with greater anthropogenic influences have reduced hatching success compared to better preserved beaches. Although we did not observe a change in the percentage of hatching among beaches, the degree of conservation did influence the survival of the nesting females. Particularly at Chenkan beach, several nesting females (six) were run-over by cars while trying to cross the road in order to nest. Nest predation was the main factor that influenced hatching success in the three studied beaches. As this factor was similar on all three beaches, the recruitment of hatchlings of both sexes to the sea did not change. However, on Punta Xen beach it was possible to observe greater predation of the nests left *in situ*, which could be related to the shortest distance between the mangrove and the beach, as well



as that beach's low level of anthropogenic impact, which allowed natural predators of turtle eggs to gain greater access to the nests.

According to the temperatures recorded in all the monitored nests, incubation temperatures did not approach the thermal limits that prevent embryonic development (25–34 °C; Howard et al. 2014). The lowest temperature recorded in the nests was 28.55 °C, while the highest temperature was 32.85 °C, which produced hatching successes of 83.84% and 81.54%, respectively. Therefore, in these locations, the incubation temperatures are still maintained within the optimum temperatures for the embryonic development of Hawksbill Turtles.

Based on gonad histology and the temperatures recorded during the TSP of nests *in situ*, the feminizing temperatures (30.21 °C and above) were determined, as well as the temperature range that produces both sexes (28.55–29.59 °C) for this population. These temperatures are similar to those reported for other populations of Hawksbill Turtles, including those obtained under controlled incubations. This is because the depth of the nests provides a buffer that prevents environmental fluctuations (Booth 2006), and the temperatures of nests *in situ* show minimal variation ( $\pm 0.07$  °C) during the TSP, remaining close to the temperature variations in experiments under laboratory conditions ( $\pm 0.05$  °C; Mrosovsky et al. 2009). Therefore, these results suggest that the use of dead hatchlings from nests *in situ* is a reliable way to estimate sex ratios among hatchling sea turtles.

According to controlled experiments in Australian Hawksbill populations, temperatures below 28.4 °C were masculinizing, while temperatures between 28.9 and 29.8 °C produced both sexes, and the temperatures that only produced females were 30.4 °C and above (Loop et al. 1995). In this study, no nests were found with exclusively masculinizing temperatures, since the lowest temperature registered during the TSP was 28.55 °C, from which most of the samples were males (83.33%), but some females were still found (16.67%). Likewise, in 1997, a study in Bahia, Brazil, found the feminizing temperature was 30.4 °C and above (Godfrey et al. 1999). However, in 1998, a study in Milman, Australia, found temperatures of 28 °C and below were masculinizing, whereas between 29.5 and 31 °C both sexes were produced, and at 32.5 °C only females were found (Dobbs et al. 2010). Those studies showed higher pivotal and feminizing temperatures compared to the present study. In general, the temperatures recorded in previous studies were similar to the results here, despite the geographical distances, as well as the differences in time.

The pivotal temperature (PT), determined according to the histological results and the TSD software, was 29.45 °C, which is close to the PT interval of other studies, even for different species, locations, and years (Hawkes et al. 2009). Specifically for *E. imbricata*, in controlled

incubations on Antigua in 1989, the PT was found to be 29.2 °C (Mrosovsky et al. 1992). Similar results were found on Milman Island, Australia, in 1998 (Dobbs et al. 2010); and in Bahia, Brazil, in 1997, the PT was reported as 29.66 °C (Godfrey et al. 1999). Therefore, it appears that the PT has remained relatively constant over the years and in different regions, which supports the hypothesis that this trait is highly conserved in sea turtles (Hawkes et al. 2009; Mrosovsky et al. 1992). This constancy makes it unlikely that the PT in sea turtles will evolve substantially in response to climate change events, which are occurring very rapidly on an evolutionary timescale (Hawkes et al. 2009), and are thus leading to feminization in many populations.

This study highlights the trend towards the feminization of the Hawksbill Turtles, at least in the western Yucatán Peninsula during 2017, since 82.05% of the monitored nests produced only female hatchlings. This trend toward increased female production was present in all monitored beaches, most remarkably in Chenkan. This feminization trend of Hawksbill Turtles has already been reported in previous studies. For example, from 1991 to 1997, in Bahia, Brazil, the sex ratio was estimated according to the incubation duration. Across six nesting seasons, the percentage was greater than 90% females (Godfrey et al. 1999). Wibbels et al. (1999) also observed a strong tendency toward feminization on Buck Island. Based on gonad histology of dead hatchlings in 51 nests *in situ*, 49 nests produced only females, while in the two remaining nests both sexes were found. Likewise, Glen and Mrosovsky (2004) reported changes in the sex ratio of Hawksbill hatchlings in Antigua, relative to those found in the work of Mrosovsky et al. (1992), which found in 1989 that sand temperatures were below the PT. When repeating this earlier work in 2003, Glen and Mrosovsky (2004) reported an increase in air temperature which increased the temperature for most of the nests above the PT. So, they proposed that in the face of climate change, the ratio of males will be reduced. In Bahia and Rio Grande do Norte, Brazil, data from 27 years of incubation durations found that 89–96% of the hatchlings were females in both locations (Marcovaldi et al. 2014).

This trend towards feminization has been documented mainly in hatchlings. However, Hawkes et al. (2013) made a summary of the sex ratios of Hawksbill Turtles and found that the bias toward feminization is greater in hatchlings than in young and adult turtles, suggesting that it may be due to high predation during this stage. Nevertheless, in a study in Anegada, a site characterized by an important foraging aggregation of Hawksbill Turtles from different areas of the Caribbean, Hawkes et al. (2013) measured the levels of testosterone and oestradiol in the blood of juvenile Hawksbill Turtles, and found 2.4- to 7.7-fold more females than males. So, between 69% and 89% of juvenile Hawksbill Turtles were females, which also shows a greater feminization during the juvenile stage. In adults and sub-adults, the sex



ratio of Hawksbill Turtles in Cuba in 1985 and 1986, was 77% females (Carrillo et al. 1998). Therefore, according to the reviewed studies, the feminization trend has been maintained for all life stages of Hawksbill Turtles in the Caribbean.

The Hawksbill Turtle population of the Yucatán Peninsula exhibits high philopatry to the nesting and breeding areas (Cuevas et al. 2012; Labastida-Estrada et al. 2019) among both females and males (González-Garza et al. 2015). The bias towards feminization can thus increase their vulnerability to inbreeding due to decreased gene flow (Hudson 1998). This trend may be exacerbated if the connection among the populations is lost due to habitat loss and fragmentation (Witherington et al. 2011), which could cause a reduction in population sizes, decreasing their genetic variability and increasing the risk of extinction (González-Garza et al. 2015; Spielman et al. 2004).

In the Yucatán Peninsula, two management units (subpopulations) are established in the Hawksbill Turtles, according to their haplotype composition. Genetically, the turtles segregate into two populations: one in the Gulf of Mexico (west of the Yucatán Peninsula) and one in Yucatán and Quintana Roo (northeast of the Yucatán Peninsula; Abreu-Grobois et al. 2003; Labastida-Estrada et al. 2019). González-Garza et al. (2015) found differences in the rates of multiple paternity between these two subpopulations of Hawksbill Turtles, with only the hatchlings from north of the Yucatán Peninsula exhibiting multiple paternity. The lack of multiple paternity among hatchlings from the Gulf of Mexico, may be a consequence of reduced mate availability (Bowen and Karl 2007; Tedeschi et al. 2014).

Hawksbill populations of the Yucatán Peninsula contain endemic haplotypes (Labastida-Estrada et al. 2019), which can be explained by historical patterns of gene flow among populations (Reece et al. 2005). The population of the Yucatán Peninsula is genetically isolated from other populations of the Caribbean, because gene flow decreases across the Caribbean populations towards the Yucatán, due to the emergence of the Campeche bank and the Florida Shelf during the Pleistocene (Reece et al. 2005). At present, in Hawksbill Turtle populations on the Yucatán Peninsula, a lower genetic variability has already been documented in the hatchlings of females that nested for the first time (neophytes) as compared to the hatchlings of remigrant females, which suggests a loss of genetic variation through time (González-Garza et al. 2015). This trend may be exacerbated by the bias toward feminization in this population of Hawksbill Turtles, as revealed in this study.

The bias towards feminization could also be further driven by the effect of climate change, since an increase in temperature even of 1–2 °C, can have considerable effects on the sex ratio (Janzen 1994). In the region of Mexico and Central America, it is estimated that environmental temperatures will exhibit an increase in thermal anomalies of 1.5 to 5 °C, due to the decrease in

precipitation between 2,000 and 2,100 mm (Christensen et al. 2007). Therefore, feminization would increase, limiting the possibility of adaptation and leading to population declines in species that are already in danger of extinction (Hamann et al. 2010; Hawkes et al. 2007; Mitchell and Janzen 2010). In addition, climate change accelerates beach erosion processes, which currently have an estimated erosion rate of 1.8 to 6.8 m/yr for the state of Campeche (Botello et al. 2010). This is driven by human infrastructure, which is the main cause of the destruction of coastal beaches (Botello et al. 2010), as well as the high deforestation that exists in the coastal dunes, severely affecting the nesting and the sex ratio of Hawksbill Turtles, so measures must be taken to reduce it.

### Conservation Implications

The trend toward feminization of Hawksbill Turtles has now been documented in several studies, including this one. This bias is predicted to increase in the face of climate change, severely affecting Hawksbill populations, which are already Critically Endangered. Therefore, according to our results, we propose the establishment of conservation strategies that promote balanced sex ratios in these turtles. Since the shade provided by the vegetation is a natural component that decreases the nest temperatures, and therefore maintains the sex ratio of Hawksbill hatchlings, priority should be given to vegetation preservation on the nesting beaches, as well as the realization of reforestation programs, especially of the native vegetation that provides greater coverage to the nests. In addition, vegetation is vital for the survival of a wide variety of other species and for maintaining the stability and integrity of the beach. In some turtle camps in Mexico, as is the case of Punta Xen, the conservation strategy is to move the nests *in situ* to hatcheries, to reduce their losses due to predation. Therefore, we recommend keeping nests *in situ*, in areas with greater vegetation cover, and carrying out continuous monitoring to reduce the predation. Or, where appropriate, we recommend the use of at least 50% artificial shade in the hatcheries, in order to provide more equitable recruitment of hatchlings in terms of their sex ratio. These measures should continue to promote the recovery of Hawksbill Turtle populations, and ensure their viability in the face of climate change.

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